Some Pharmacologic and Toxicologic Effects of Di-2-ethylhexyl Phthalate (DEHP) and Other Plasticizers*

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With the recent demonstration of the migration of phthalate ester plasticizers from vinyl plastic biomedical devices *(1—4)*, its identification in human and animal tissues *(5, 6)*, and evidence for its ubiquitous distribution in the environment *(7, 8)*,†† it has become necessary to reevaluate the toxicologic potential of this class of chemical compounds. Di-2-ethylhexyl phthalate (DEHP), as an example of the most widely used of the phthalate esters, deserves particular emphasis. In general, the phthalate esters have been reported to have a low order of acute toxicity *(9—11)*. For example, of eight different phthalate esters examined in one study, the intraperitoneal LD_{50} dose in mice ranged from 1.5 to 14.2 g/kg. In rats, the intraperitoneal LD_{50} of DEHP has been reported by several investigators to range from 2 to 31 g/kg. One report by Hodge in 1943 *(9)* indicated that a single intraperitoneal dose of 128 g/kg of DEHP produced death in only 5% of a group of treated mice. Other studies on the oral administration of DEHP indicated an LD_{50} of approximately 30 g/kg in rats and rabbits given a single oral dose. Ninety-day and 2-year feeding studies in rats and 1-year feeding studies in guinea pigs and dogs likewise indicated a low order of toxicity for DEHP. These data have resulted in the Food and Drug Administration approving DEHP for use in plastic wrapping for food intended for human consumption *(12)*.

The major shortcoming of the available toxicologic data is that they deal primarily with gross, overt effects, i.e., morbidity, mortality, changes in body and organ weights, and histologic changes. They do not take into account more subtle toxicologic effects. [Examples of the subtle toxic effects of vinyl plastics additives have been given in the literature *(13—16)*.] This paper presents such subtle effects observed in our laboratory.

Results

Effect of DEHP on Response to Drugs

It has been well documented that xenobiotics (drugs, environmental chemicals, etc.) are metabolized in the liver and that

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such metabolism determines to a large extent the duration of response to that chemical in the body. Furthermore, it has been shown that a wide variety of chemicals are able to alter this metabolic system *(17)*.

Experiments were undertaken to determine if acute treatment with phthalate esters would alter the response of rats and mice to a commonly employed test drug, hexobarbital. The duration of the anesthetic effect of the barbiturate, referred to here as "sleeping time," was determined. Male mice, of body weights in the 20–22g range, were treated with either 250 mg/kg or 500 mg/kg of butylglycolyl butyl phthalate (BGBP) or di-2-ethylhexyl phthalate (DEHP). The phthalates were prepared as emulsions in 3% acacia in physiological saline as the vehicle. Control mice received appropriate volumes of the acacia vehicle. Thirty minutes after administration of the phthalate or vehicle, hexobarbital was administered intraperitoneally at a dose of 60 mg/kg. The duration of barbiturate-induced sleep was taken from the time of injection of hexobarbital to the time the animal regained his righting reflex. The results are shown in Figure 1. It can be seen that both doses of DEHP produce a significant increase in sleeping time over control values, the higher dose producing approximately a doubling in sleep time. BGBP was correspondingly less effective. The lower dose produced no significant effect, but the higher dose showed a significant prolongation, though less than with the same dose of DEHP.

A comparable effect of DEHP was seen in rats. The results are shown in Figure 2. In these experiments, male rats weighing 200–250g, were given DEHP intraperitoneally at a dose of 500 mg/kg as an emulsion in acacia. As with the mice, hexobarbital at a dose of 250 mg/kg was given 30 min after administration of the phthalate or vehicle. It can readily be seen that DEHP, at the dose used, causes only a slight though significant increase in hexobarbital sleeping time. Shown along the bottom portion of the figure are the blood levels of hexobar-

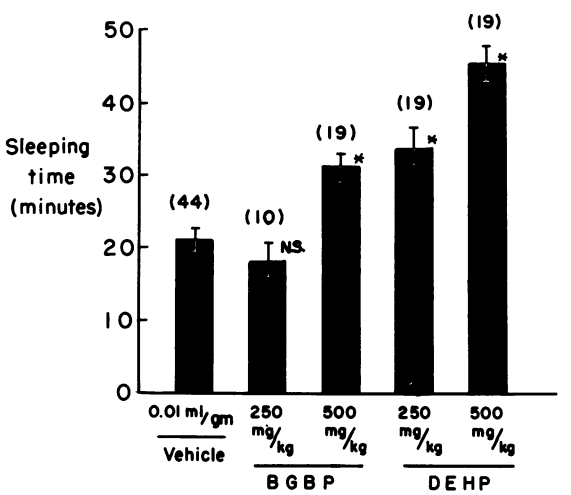

FIGURE 1. Effect of BGBP and DEHP on hexobarbital sleeping time in mice. The asterik indicates responses that are significantly different ($P < 0.05$) from vehicle-injected controls. NS= not significant ($P > 0.05$). The numbers in parentheses are the number of animals tested per group. Brackets represent ± 1 standard error.

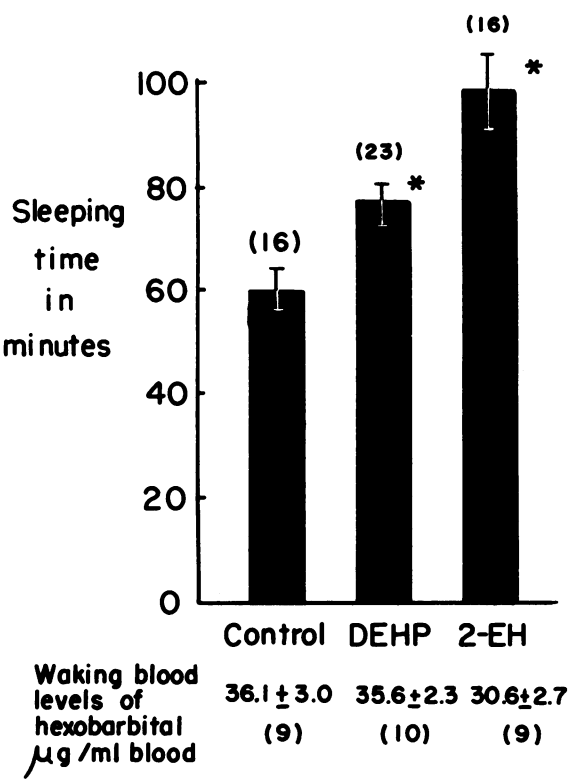

FIGURE 2. Effect of DEHP and 2-ethylhexanol (2-EH) on hexobarbital sleeping time in rats. Asteriks, brackets, and numbers in parentheses are the same as in Figure 1.

bital determined in samples drawn immediately upon the animal awakening. It can be seen that, although there is a prolongation of pharmacologic response to hexobarbital, this effect is not produced by an increased sensitivity of the central nervous system to the depressant effects of the barbiturate. This is indicated by the fact that the rats treated with DEHP awoke when the concentration of hexobarbital in the blood reached the same level at which the controls awoke. Also shown in Figure 2 are the results obtained when the rats were pretreated with an equimolar concentration of 2-ethylhexanol (2-EH), a known anesthetic that represents a possible metabolite of DEHP. The results seen with 2-EH are consistent with the possibility that the de-esterified product of DEHP might account for the ability of the ester to prolong barbiturate anesthesia. Although there is a trend with 2-EH for an increased degree of sensitivity of the CNS to hexobarbital, the lowered blood level of the barbituate is not significantly different from that seen in the controls.

The ability of DEHP to prolong hexobarbital sleeping time *in vivo* without any significant alteration in the waking blood levels of the barbiturate suggests that the effect is related to a decreased rate of disappearance of the test drug from the blood. Since a major route for the elimination of this drug from the body is via hepatic metabolism, the effect of DEHP on the rate of hexobarbital metabolism in the isolated, perfused rat liver was studied. The results are seen in Figure 3. DEHP was added to the perfusate at an initial level of 70 μg/ml. This level was established prior to installation of the isolated liver. The DEHP–containing perfusate was then allowed to circulate through the liver for 2 hr, after which time hexobarbital was added to the main perfusate reservoir. The level of hexobarbital in the reservoir was then determined over the next 30 min of perfusion. It can be seen from the figure that the barbiturate disappeared logarithmically in this preparation and that the rate of disappearance was not

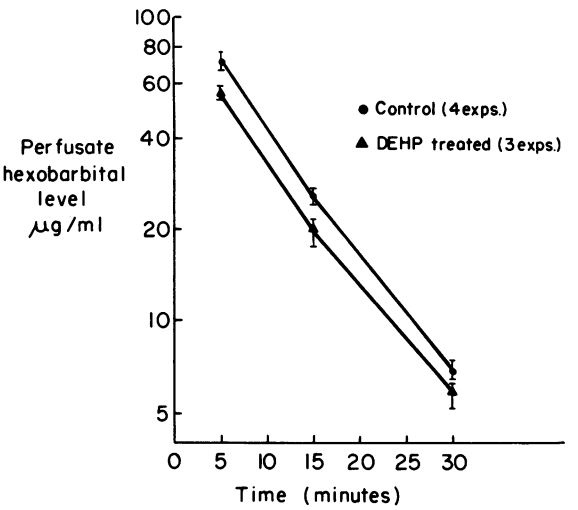

FIGURE 3. Rate of disappearance of hexobarbital in the isolated rat liver perfusion system.

significantly altered by DEHP. However, at each time point the hexobarbital level in the perfusate was significantly lower in the DEHP-treated system. Presumably, this is related to a more rapid initial distribution of the barbiturate into the liver followed by no significant alteration in its rate of metabolism. This experiment thus rules out an effect of DEHP on the rate of hepatic metabolism as an explanation for its ability to prolong hexobarbital sleeping time. The data, on the other hand, suggest the possibility for an effect on the distribution of the drug into various organs. Such barbiturate distribution measurements have not yet been made. Though the exact mechanism is not known, it is clear that BGBP and DEHP can alter the pharmacologic response to a barbiturate.

Behavioral Effects

Behavioral effects were evaluated in two different systems: (a) self-stimulation of the hypothalamic "pleasure center" and (b) spontaneous running in a metered activity wheel. In the former system, permanent electrodes were stereotaxically implanted in the hypothalamic pleasure center of the rat's brain. Following recovery from the surgical procedure, the rat was taught that by pressing a bar in a test cage, he would be able to

activate the electrodes and thus receive a "pleasureable" stimulus. After a short period of training, each rat displayed a rather characteristic rate of bar-pressing activity during a daily 1-hr test period. The effect of an intraperitoneal dose of 500 mg DEHP/kg on rats in this test system is shown in Figure 4. The DEHP was administered 2 hr before the rats were placed in the test situation. The data are presented as the mean per cent of the control rate of bar pressing. It can readily be seen that the intraperitoneal administration of the acacia vehicle slightly, but nonsignificantly, decreased the bar-pressing rate. DEHP, on the other hand, depressed the rate to 30% of control.

Figure 5 shows the results of the study on the free-running activity of the rats. Animals were maintained in individual cages having access to a freely rotating drum on which was mounted a mechanical metering device for recording the numbers of revolutions. DEHP (500 mg/kg) or vehicle was administered at 12 noon, and the running activity was monitored during the following 24-hr period. It can be seen that injection of the vehicle alone significantly altered this behavioral activity from control levels. However, it

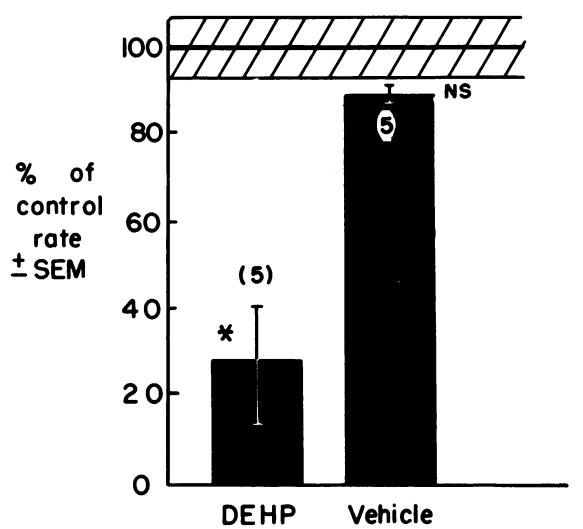

FIGURE 4. Effect of DEHP on hypothalamic self-stimulation in rats. The cross-hatched area indicates the control rate ± 1 standard error and is expressed as 100. The experimental data is expressed as the mean of the per cent change from control observed for each animal.

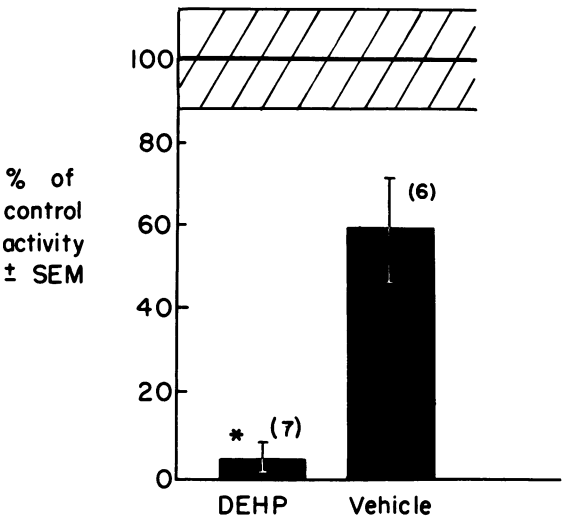

FIGURE 5. Effect of DEHP on the 24-hr free-running activity of rats. Data expressed as in Figure 4.

is clear from the data that DEHP virtually eliminated all running activity during the subsequent 24-hr period.

In spite of these behavioral deficits elicited by DEHP, it is important to note that animals appeared not to be in a narcotized or anesthesized state. They appeared to be reasonably alert to external stimuli.

Effect on Recticuloendothelial Function

Unpublished studies *(2)* have indicated the accumulation of DEHP in those organs having a high degree of reticuloendothelial function, i.e., liver, spleen, and lungs. For this reason, experiments were undertaken to evaluate the effect of DEHP on reticuloendothelial activity in the intact animal. The system chosen for study was the clearance of colloidal carbon particles from the blood of intact rats. In these experiments rats were anesthesized with pentobarbital and given an intravenous dose of colloidal carbon (40 mg/kg). At 2-min intervals blood samples were drawn from the tail vein and assayed for colloidal carbon. The results are shown in Figure 6, where it can be seen that in vehicle-treated controls the colloidal carbon clearance is logarithmic with a half-time of approximately 4 min. Intravenous admini-

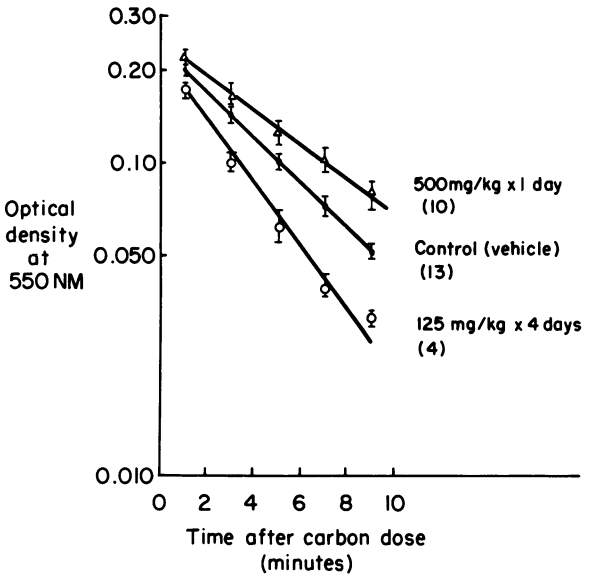

FIGURE 6. Effect of acute and subacute intravenous doses of DEHP on *in vivo* reticuloendothelial function in rats. Each data point is the mean ± 1 standard error for the number of animals indicated in parentheses.

stration of a single dose of 500 mg of DEHP/kg 24 hr prior to the test dose of carbon resulted in a significant depression in the clearance rate. On the other hand, four injections of 125 mg of DEHP/kg each, given on alternate days with the carbon clearance test given 24 hr after the last of the four doses, resulted in a significant increase in the rate of carbon clearance. Thus, depending on the schedule of administration, DEHP can either depress or stimulate reticuloendothelial function.

Relationship of DEHP to Microaggregation of Platelets in Stored Blood

In addition to any direct toxicologic effects of DEHP on the intact animal is the problem of evaluating the effect of this compound (or class of compounds) on the quality of blood stored in vinyl plastic bags for transfusion. One of the major drawbacks of stored blood is the progressive accumulation of microembolic aggregates which carry a threat of occluding the capillary beds of the transfusion recipient. Swank *(18)* has demonstrated that these aggregates consist of clumps of platelets which form because of an increasing degree of platelet adhesiveness. Based on unpublished observations from this laboratory on the high affinity of stored platelets for DEHP *(2)*, it became of interest to determine the relationship between the DEHP content of stored blood and the extent of microaggregate formation.

A direct measure of microaggregate formation is available by measurement of the pressure generated across a filtration screen of given porosity when blood is pumped across that screen at a constant flow. The pressure, referred to as screen filtration pressure (SFP), measures the resistance to flow, and as such is a measure of the occlusion of the pores and the adhesiveness that the occluding particles have for one another. The procedure is essentially that described by Swank *(19)*.

Dog blood was stored in commercial vinyl plastic storage bags for periods of time up to 21 days. At varying periods of time, aliquots were removed aseptically, and assayed for screen filtration pressure and DEHP content. The correlation of these two parameters are shown in Figure 7. A highly significant correlation coefficient r of + 0.84 was found between the degree of microaggregation as measured by screen filtration pressure and the content of DEHP. It should be emphasized, however, that a correlation does not imply a cause and effect relationship. These results, indicating a significantly high degree of correlation, merely point out the need for further direct studies of the effect of DEHP on platelet aggregation.

Effect on Embryonic Heart Cells Maintained in Tissue Culture

In experiments on beating chick embryo heart cells maintained in tissue culture, performed in collaboration with Dr. Robert DeHaan of the Carnegie Institute of Washington, it was observed (Table I) that DEHP at levels of 4 μg/ml in the tissue culture media resulted in the complete cessation of function. Within 24 hr, some 97–98% of the cells were dead and undergoing autolysis. Another type of plasticizer, di-2-ethylhexyl adipate (DEHA) at a level of

Table 1. Effect of plasticizers on beating chick heart cells maintained in tissue culture.

Plasticizer	Concentration in culture medium, μg/ml	Beating heart cells at 30 min, % of control cultures	Viable heart cells at 24 hr, % of control cultures
DEHP	4.0	2 - 3	2 - 3
DEHA	1.5	50	100

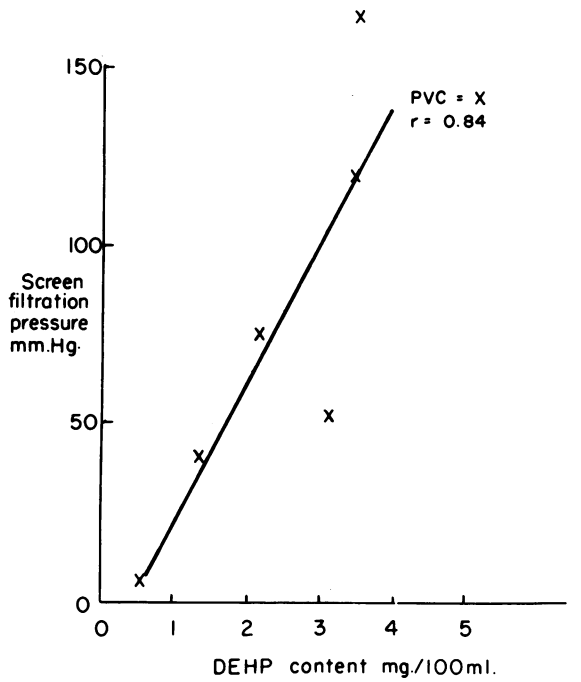

FIGURE 7. Correlation of DEHP content of dog blood stored in PVC plastic bags and the presence of microaggregates as measured by screen filtration pressure.

1.5 μg/ml reduced the number of beating cells to 50% of control levels, but essentially all the cells were viable after 24 hr.

Conclusions

Previous published data on the toxicology of the phthalate esters (including DEHP) have indicated a low order of toxicity. However, the results presented here indicate that, under the appropriate conditions, DEHP can be demonstrated to have significant effects on a variety of biological systems. These effects would not be observed, for the most part, in the classical toxicologic evaluations upon which the relative safety of plasticizers have been previously based.

Of particular interest is the sensitivity of embryonic heart cells to DEHP. A level of 4 μg/ml in the culture media is lethal to 97–98% of these cells. It should be pointed that this level of DEHP is one that is reached in human blood stored in vinyl plastic bags after a period of 1–2 days of storage (*2, 5*). Also, of concern in this regard is the recent report of Nazir et al. *(6)* of the localization of DEHP in beef heart mitochondria.

In general, it is felt that the results presented in this paper warrant a further detailed evaluation of the potential toxicity of those additives such as plasticizers which have a high tendency for migration from the finished product. These continuing evaluations will be necessary until technology yields a usable vinyl plastic subsitute from which migration of additives, under conditions of use, is negligible.

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